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Inhibition of myocardial apoptosis reduces infarct size and improves regional contractile dysfunction during reperfusion

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Abstract

Objective: Myocardial apoptosis is primarily triggered during reperfusion (R) through various mechanisms that may involve endonuclease to cleavage genomic DNA in the internucleosomal linker regions. However, the relative contribution of myocardial apoptosis to development of myocardial injury during R remains unknown. In the present study, we examined whether inhibition of apoptosis with aurintricarboxylic acid (ATA), an endonuclease inhibitor, during R reduces infarct size and improves regional contractile function. **Methods and Results:** In two groups of chronically-instrumented dogs, 1 h of left anterior descending (LAD) coronary occlusion was followed by 24 h of R with infusion of saline (control, $n=8$) or ATA (1 mg/kg/h, $n=8$) into the left atrium starting 5 min before R and continuing for 2 h. ATA significantly reduced apoptotic cells (TUNEL staining) in the peri-necrotic myocardium (12±1%* vs. 36±4%), consistent with the absence of DNA laddering. To confirm inhibition of apoptosis with ATA, densitometrically, Bcl-2 (% of normal myocardium) was significantly increased vs. control (102±12* vs. 68±9) and Bax as well as the activated caspase-3 were significantly reduced vs. control (108±17* vs. 194±42 and −29±4* vs. 174±43, respectively). ATA significantly improved segmental shortening (3.3±1.2* vs. −1.8±0.7%) and segmental work (79.3±11.3* vs. 7.1±5.8 mmHg/mm) in area at risk myocardium, and reduced infarct size (TTC staining, 27±0.2* vs. 37±0.5%), confirmed by lower plasma creatine kinase activity. In addition, myocardial blood flow (0.9±0.1* vs. 0.4±0.1 ml/min/g) and endothelial-dependent maximal vascular relaxation (119±6* vs. 49±8%) were significantly improved. Myeloperoxidase activity in area at risk myocardium, a marker for neutrophil accumulation, was also significantly reduced (17±4* vs. 138±28 ΔAbs/min). **Conclusions:** These data suggest that the inhibition of apoptosis during R is associated with a reduction in infarction, improvement in regional contractile and vascular endothelial functions as well as augmentation in myocardial blood flow. *$P<0.05$ vs. control group.

Keywords: Apoptosis; Infarct size; Myocardial contraction; Reperfusion

1. Introduction

Necrosis and apoptosis are two major distinct types of cell death in myocardium that have been linked with reperfusion (R)-induced myocardial injury after reversible coronary occlusion [1,2]. It has been demonstrated that in rabbit, rat and dog models of regional ischemia and R, myocardial apoptosis was primarily triggered or accelerated during R [3–5]. A clinical study also found that the appearance of myocardial apoptosis was significantly accelerated in reperfused myocardium [6]. Inhibition or interruption of the execution phase of apoptosis with caspase inhibitors has shown a reduction in infarct size following reversible coronary occlusion [7,8]. However, the relative contribution of apoptosis to infarct extension and regional contractile dysfunction during a longer period of R remains unknown. Recently we demonstrated that consistent with extension of infarct size and attenuation of

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regional contractile function after coronary occlusion [9], myocardial apoptosis in the peri-necrotic myocardium progressively developed from early to late phase of R, suggesting that apoptosis may participate in exacerbation of myocardial injury [10]. It is conceivable, therefore, that apoptotic cells detected from the peri-necrotic myocardium may undergo secondary necrosis, and that the extension of infarction may in part be elicited from the delayed apoptosis during R. If the end stage of R injury is modulated by apoptosis, is there any 'window of opportunity' that intervention can stop this suicide program? Does the inhibition of apoptosis during R lessen extension of infarction and improve regional contractile function after myocardial ischemia? Since apoptosis represents a potentially preventable form of cell death due to its active and regulated natures, it is likely that inhibition of apoptosis may help to reduce myocardial injury during R.

Aurintricarboxylic acid (ATA), an endonuclease inhibitor known to inhibit apoptosis from in vivo and in vitro studies [11–14], has shown a profound effect on infarct size in gerbils when this compound was intraventricularly administered before transient brain ischemia [12]. The purpose of this study, therefore, was to use ATA as a tool by its potent anti-apoptotic character to test whether exogenous ATA limits the extension of infarct size and improves regional contractile and endothelial function in a dog model following extended R.

2. Methods

2.1. Surgical preparation of animals

Studies were conducted according to the guidelines of the committee on the Animal Welfare Act and Emory University Veterinary policies and the *Guide for the Care and Use of Laboratory Animals* of the Institute of Laboratory Animal Resources, National Council Department of Health and Human Services publication no. [NIH] 85-23, revised 1985.

Dogs of either sex were used in the study. All animals were initially anesthetized with intramuscular morphine sulfate (4 mg/kg). A bolus injection of pentothal (20 mg/kg) was given followed by continuous inhalation of isoflurane (0.5–2% in oxygen) after endotracheal intubation. A left lateral thoracotomy was performed, and micromanometer pressure transducers were inserted into the left internal mammalian artery and ventricle to monitor aortic and ventricular pressure. A pair of ultrasonic crystals was implanted in the anterior-midmyocardium to measure regional contractile function. A cuff-type flow probe (Pulsed Doppler Flowmeter, Triton Technology, San Diego, CA, USA) was placed below the first diagonal branch of LAD and the ligating snare for measurement of coronary artery flow [15]. A catheter was inserted into the left atrium for injection of colored microspheres to measure regional myocardial blood flow. A plastic snare was placed below the first diagonal branch of LAD to reversibly produce a zone of regional ischemia in the left ventricle. After 2 h of R, the thoracotomy incision was closed in layers and a regimen of broad spectrum antibiotics and analgesia was initiated throughout the R period.

2.2. General experimental protocol

Dogs were randomly divided into two groups. Either saline (control, $n=8$) or aurintricarboxylic acid (ATA from Sigma, 1 mg/kg/h, $n=8$) was infused through the left atrium at a rate of 1 ml/min beginning 5 min before R and ending 2 h after R. All dogs underwent 1 h of LAD coronary occlusion and 24 h of R. In both groups, hemodynamic and regional contractile function were measured at baseline, ischemia, 2 h and 24 h of R. At the end of the experiment, segments of the LAD and left circumflex (LCX) coronary arteries were isolated and used to evaluate agonist-stimulated vascular endothelial response. Tissue samples from the non-ischemic and ischemic zones were used to quantify myocardial apoptosis, infarct size, myocardial blood flow and neutrophil accumulation (see below). An additional six dogs were used as sham control to observe the effect of ATA on the heart after surgery. In these animals, ATA was administered at a dose of 1 mg/kg/h for 2 h and the chest was closed for 24 h after completing surgical procedures without coronary occlusion.

2.3. Tissue preparation

Myocardial tissues (150–200 mg for each) from non-ischemic and ischemic zones were kept in tubes for DNA isolation and Western blot assay. For detection of apoptosis, the transmural ischemic tissue was embedded in optimal cutting temperature compound (O.C.T., Sakura Finetek), frozen in liquid nitrogen and then stored at −70 °C. Cryosections (7 μm thick) from frozen tissue were then obtained using a Hacker–Bright cryostat and thaw-mounted onto Vectabond (Vector Laboratories, Burlingame, CA, USA) coated slides or Fisher-Plus (Fisher Scientific) slides, refrozen, and stored at −70 °C with desiccant until use.

2.4. The detection of myocardial apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-X nick end labeling (TUNEL)

The TUNEL signal in the tissue sections were determined using an in situ cell death detection kit (Boehringer Mannheim, Ridgefield, CT, USA) as described previously [4]. Briefly, DNA fragments were detected by an anti-fluorescein antibody conjugated with alkaline phosphatase, a reporter enzyme that catalytically generates a red-colored product from Vector Red substrate. The slides

were counterstained with hematoxylin, dehydrated in graded alcohols and covered with Accumount medium. For each slide, color video images of 10 separate fields were captured randomly and digitized by using a ×20 objective on an Olympus IX50 microscope connected to an IBM computer. The apoptotic cells were calculated as percentage of TUNEL-positive cells from total cell nuclei [3].

2.5. The presence of DNA ladder in agarose gel electrophoresis

Appearance of a DNA ladder was detected using agarose gel electrophoresis as described previously [3]. Briefly, frozen tissue samples from non-ischemic and ischemic zones were minced in lysis buffer and were quickly homogenized with a microfuge tube pestle. The tissue was digested with proteinase K and incubated with RNase A. Supernatants containing DNA were precipitated with isopropanol and the resulting DNA pellets after centrifugation were washed with ethanol and dissolved in DNA hydration solution. DNA electrophoresis was carried out and DNA ladders were visualized under ultraviolet light.

2.6. Western blot analysis of Bcl-2, Bax and caspase-3 proteins

Determination of Bcl-2, Bax and caspase-3 proteins by Western blot assay was primarily described previously [3]. Briefly, tissue samples from non-ischemic and ischemic zones were homogenized with lysis buffer and protein concentration was measured by the DC Protein Assay (Bio Rad). Proteins (80 μg) for each lane were mixed with loading buffer, and loaded onto gradient SDS–polyacrylamide gel. Proteins kept in the nitrocellulose filter after transfer were blocked with milk and the membranes were then exposed to antibodies (Santa Cruz, CA, USA) including rabbit polyclonal anti-human Bcl-2 (ΔC21), rabbit polyclonal anti-human Bax (P19) and rabbit anti-human caspase-3 (H277), respectively. Bound antibody was detected by horseradish peroxidase conjugated anti-rabbit IgG. Finally, ECL detection reagents were employed to visualize peroxidase reaction product. The density at specific molecular weights was measured by NIH image analysis software on a Power Macintosh.

2.7. Determination of area at risk and infarct size

At the end of the experiment, Unisperse blue dye was injected into the aortic root to stain the normally perfused region blue and outline the area at risk. The area at risk was then separated from the non-ischemic zone and incubated in a solution of 1% triphenyltetrazolium chloride (TTC) to differentiate necrotic (pale) from non-necrotic area at risk tissue. The gravimetric method was used to quantify infarct size [3].

2.8. Plasma creatine kinase (CK) activity

Arterial blood samples were withdrawn at baseline, at the end of ischemia, 2 and 24 h of R to measure CK activity. The plasma was drawn off after centrifugation and CK activity was analyzed spectrophotometrically according to the method of Rosalki (Sigma Diagnostics). Plasma CK activity was expressed as IU/mg protein [3].

2.9. Determination of regional myocardial blood flow

Colored microspheres were used to quantify myocardial blood flow. Samples of non-ischemic, ischemic subepicardial and subendocardial myocardium were placed in tared vials marked according to their anatomical location. Tissues and reference blood samples were analyzed in a Spectra Max Spectrophotometer (Molecular Devices) as reported previously [9]. Results are expressed as ml/min/g tissue.

2.9.1. Postischemic vascular ring reactivity

LAD and LCX coronary artery segments were isolated and placed into Radnoti tissue baths. After the coronary rings were preconstricted with thromboxane A_2-mimetic U46619, and subsequently dilated in concentration–response fashion with the endothelium-dependent vasodilator, acetylcholine, and the endothelium-independent vasodilator, sodium nitroprusside (SNP) in incremental concentrations. Responses to vasodilators were analyzed using a videographics program developed in our laboratory [9].

2.9.2. Measurement of myeloperoxidase activity (MPO) in cardiac tissue

Tissue samples from non-ischemic and ischemic zones were homogenized. After centrifugation, the supernatants were decanted and mixed with O-dianisodine dihydrochloride and H_2O_2 in phosphate buffer. The change in absorbance was measured spectrophotometrically at 460 nm. MPO activity was expressed as Δabsorbance units/min [9].

2.9.3. Statistical analysis

Concentration response curves of vascular relaxation were calculated as a percentage of U46619-induced increase in isometric force. Student's *t*-test was used to analyze differences between such parameters as vascular responses, MPO, myocardial blood flow and infarct size data. Hemodynamic, regional contractile function and other time-dependent determinations were analyzed by repeated analysis of variance. A P value less than 0.05 was accepted as statistically significant.

3. Results

3.1. Time course of hemodynamic changes

Hemodynamic data for heart rate (HR), mean aortic pressure (MAP), left ventricular systolic pressure (LVSP), dP/dt_{max}, left ventricular end-diastolic pressure (LVEDP), and LAD coronary artery blood flow (CBF) in all groups are shown in Table 1. There were no differences between groups for any of the hemodynamic parameters at baseline. Coronary occlusion caused a significant increase in HR. At 24 h of R, HR tended to be less in the ATA group, but it did not reach a significant difference compared with the control group. MAP in the control group at 24 h of R was significantly reduced, however, it remained consistent throughout the procedure in the ATA group. There were no group differences in LVSP, LVEDP, positive and negative dp/dt during the course of experiment. ATA treatment significantly increased LAD blood flow compared with the control group during 2 h of R. Surgical operation without coronary occlusion did not induce hemodynamic change in the sham group.

3.2. Regional contractile function

Regional contractile function data for systolic shortening (SS) and segmental work (SW) in the area at risk are shown in Table 1 and Fig. 1. There was no difference on any measured parameters at baseline. During ischemia, paradoxical systolic expansion was exhibited in both groups with a rightward shift in the pressure–length relationship. There was a significant decline in SS and SW. At 2 h of R, SS and SW in the ATA group tended to be greater than that in the control group, but it did not reach significance. Wall motion remained dyskinetic throughout the entire period of R in the control group. However, significant recovery in SS and SW was detected in the ATA group at 24 h of R, suggesting an improvement in regional contractile function. No change in regional contractile function was found in the sham group.

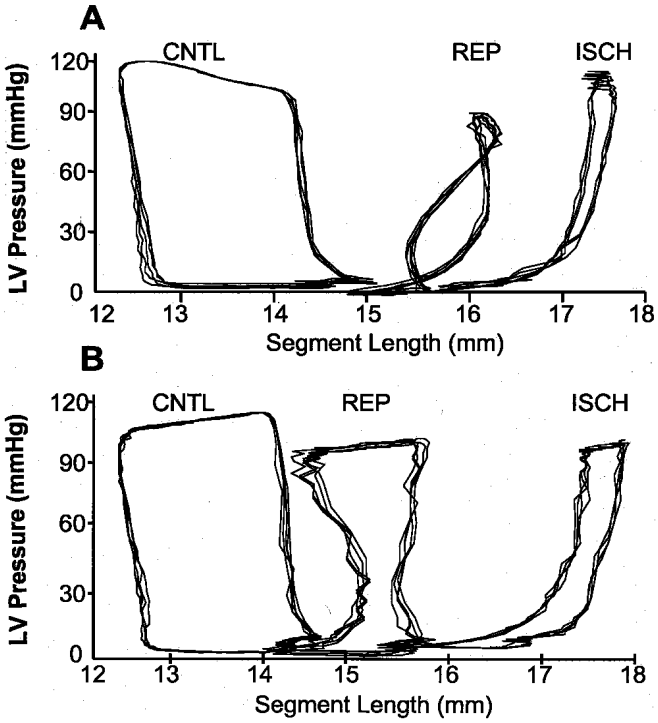

Fig. 1. Examples of instantaneous LV pressure–segment length loops for a representative experiment in control (A) and ATA (B) groups. ATA treatment significantly improved systolic shortening at the end of reperfusion (R). Segmental work was calculated as the integral of each pressure–length loop. CNTL, baseline time point; ISCH, end of ischemia; REP, 24 h of R.

Table 1
Hemodynamics and regional contractile function during the course of the experiment

Time	Group	Parameters								
		HR	MAP	LVSP	LVEDP	$+dp/dt$	$-dp/dt$	CBF	SS	SW
Baseline	Control	87±8	94±6	101±6	8±2	1473±82	−1413±84	16±1	15.8±1.2	148±18
	ATA	86±3	105±4	109±3	10±1	1535±186	−1459±50	13±1	18.2±1.1	186±9
	Sham	87±6	97±6	101±6	9±1	1452±68	−1405±54	14±2	15.1±1.2	139±14
Ischemia	Control	109±8*	101±5	103±6	13±2	1443±61	−1432±91	–	−2.3±0.6*	32.6±9.6*
	ATA	104±4*	100±4	105±3	16±1	1459±26	−1367±51	–	−0.2±0.4*	41±5.7
	Sham	86±7	98±7	102±5	9±2	1443±59	−1402±82	14±3	15.2±1.1	139±13
R2h	Control	114±6*	84±3	90±4	9±2	1369±66	−1280±93	14±1	−1.5±0.4*	16±4.6*
	ATA	111±4*	95±3	95±3	13±2	1374±60	−1300±62	22±1*†	1.3±0.9*	63±9*
	Sham	89±8	99±7	101±5	10±2	1449±49	−1414±67	15±1	15.6±0.8	140±11
R24h	Control	131±7*	77±2*	84±4	9±2	117±104	−1088±104	7±1*	−1.8±0.8*	7.31±5.8*
	ATA	119±6*	95±3†	98±5†	12±1	1242±92	−1259±105	12±1	3.3±1.2*†	79.3±11.3*†
	Sham	99±9	101±7	103±6	9±2	1467±61	−1441±68	16±2	14.6±0.8	135.12

HR, heart rate (beats/min); MAP, mean aortic pressure (mmHg); LVSP, left ventricular peak systolic pressure (mmHg); LVEDP, left ventricular end diastolic pressure (mmHg); positive dp/dt (mmHg/s); negative dp/dt (mmHg/s); CBF, mean LAD flow (ml/min); SS, systolic shortening (%); SW, segmental work (mmHg/mm); R2h and R6h, 2 and 6 h of reperfusion, respectively. Sham ($n=6$): the chest was closed for 24 h after surgical procedures with coronary occlusion. All values are expressed as mean±S.E.M. *$P<0.05$ vs. baseline value; †$P<0.05$ vs. control group.

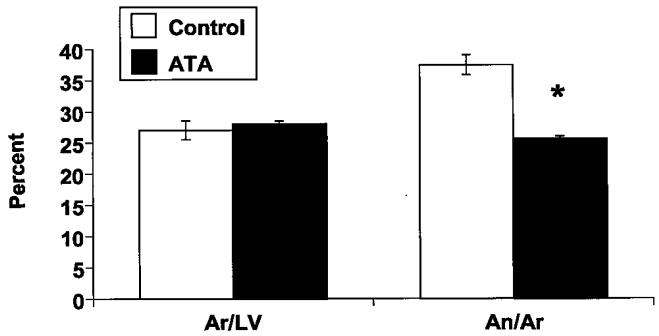

Fig. 2. The area at risk relative to the left ventricle (Ar/LV) and infarct size relative to the area at risk (An/Ar) after 1 h ischemia followed by 24 h of R. ATA treatment significantly reduced infarct size after 24 h of R compared with the control group. Bars represent group mean; brackets indicate S.E.M. *$P<0.01$ vs. control.

3.3. Reduction in extension of necrosis

The area placed at risk by coronary occlusion expressed as a percent of the left ventricular mass (Ar/LV), and the area of necrosis expressed as a percent of the area at risk (An/Ar) are shown in Fig. 2. Ar/LV was comparable in the two groups ranging between 25–28% in the control group and 26–29% in the ATA group. Administration of ATA at R significantly reduced An/Ar by 33% compared with that in the control group at 24 h of R. No necrosis was detected in the sham group.

3.4. Creatine kinase activity during the course of the experiment

There was no group difference in plasma CK activity at baseline. Coronary occlusion only slightly increased CK values with no group differences. CK activity was significantly increased from 2 to 24 h of R relative to ischemia, reaching values (IU/g protein) of 19±3 and 40±4, respectively, in the control group, suggesting an extension of infarct size during R. However, CK activity was significantly attenuated in the ATA group at these time points with 10±1 and 19±2, $P<0.01$, respectively. There was no difference in plasma CK activity after the chest was closed (1.2±3) for 24 h compared with baseline value (0.8±2) in the sham group.

3.5. Detection of TUNEL-positive cells in the peri-necrotic myocardium

No TUNEL-positive cells were found in the non-ischemic zone in either group. As shown in Fig. 3, however, the number of TUNEL-positive cells expressed as a percent of total normal nuclei was significantly increased in the peri-necrotic zone in the control group (36±4%, Fig. 3B) relative to the non-ischemic zone, which was consistent with the appearance and increased intensity of DNA ladders in this group (see below). ATA administered at R significantly reduced TUNEL-positive cells in the peri-necrotic zone (12±1%) versus the control group ($P<0.01$), consistent with disappearance of DNA laddering in this group (see below). No clear TUNEL-positive staining was detected in the sham group.

3.6. DNA fragmentation

DNA nucleosomal fragmentation of myocyte in non-ischemic and peri-necrotic zones in both groups is shown in Fig. 4. No visible DNA 'ladders' were found in the non-ischemic zone in either group. In contrast, genomic DNA isolated from the ischemic zone in the control group produced a typical 'ladder' pattern from all eight animals after ischemia and R, consistent with induction of TUNEL-positive cells in this area. Treatment with ATA significantly decreased the appearance of DNA 'ladders' in the peri-necrotic zone. One out of eight animals from this

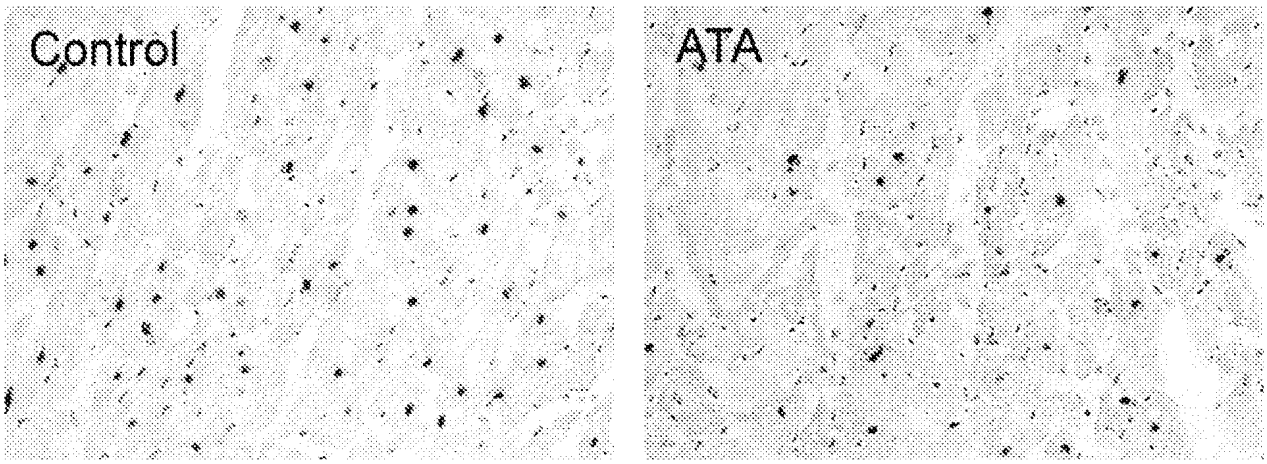

Fig. 3. Detection of apoptotic myocytes in the peri-necrotic myocardium by TUNEL staining after 1 h ischemia followed by 24 h of R. Relative to the control group, the number of TUNEL-positive cells was significantly decreased by ATA treatment. TUNEL-positive cells were not detected in the posterior and non-ischemic zones after ischemia and R. Red staining indicate TUNEL-positive cells (magnification ×200).

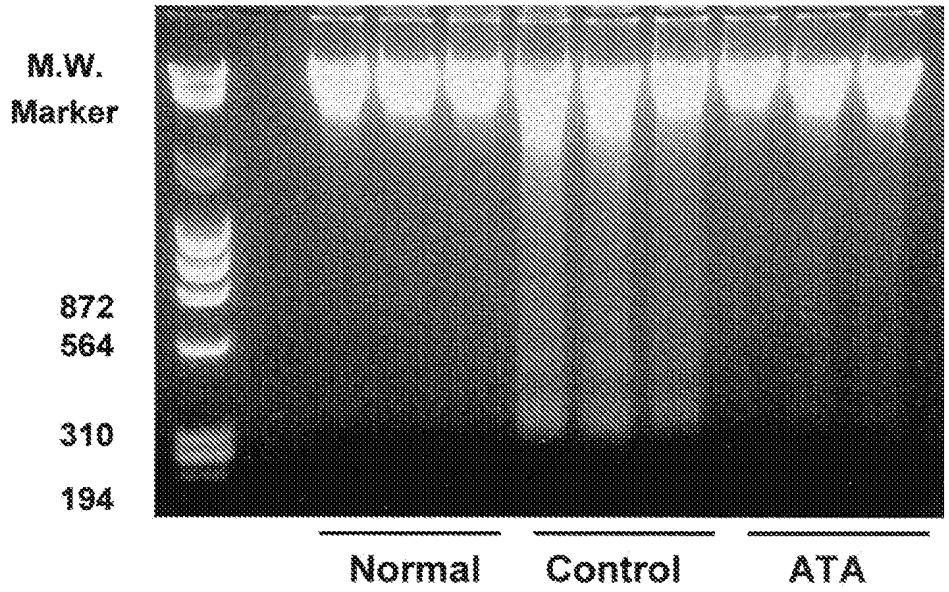

Fig. 4. DNA fragmentation in ischemic myocardium after 1 h ischemia followed by 24 h of R. Lanes 1–3 represent normal tissue; 4–6 represent ischemic tissue in the control group and 7–9 represent ischemic tissue in ATA group. This picture is representative of eight samples per group.

group showed mild DNA laddering. No apparent DNA 'ladders' were found in the sham group.

3.7. Expression in Bcl-2, Bax and caspase proteins after ischemia and R

Expression of Bcl-2 and Bax proteins in non-ischemic and peri-necrotic zones was visualized by Western blot analysis as shown in Fig. 5. Bcl-2 was clearly expressed in the non-ischemic zone. Densitometrically (% normal tissue), coronary occlusion and R significantly reduced Bcl-2 expression and increased Bax expression compared with non-ischemic tissue, respectively (Fig. 5). Treatment with ATA was associated with greater Bcl-2 and attenuated Bax expression relative to the control group. Western blot analysis using polyclonal caspase-3 antibody recognized both pro- and active caspase-3. As shown in Fig. 6, procaspase-3 was present in normal tissue in both groups. Consistent with increased number of TUNEL-positive cells after ischemia and R, active caspase-3 was only present in apoptotic lysates collected from peri-necrotic myocardium in the control group. ATA treatment at R significantly

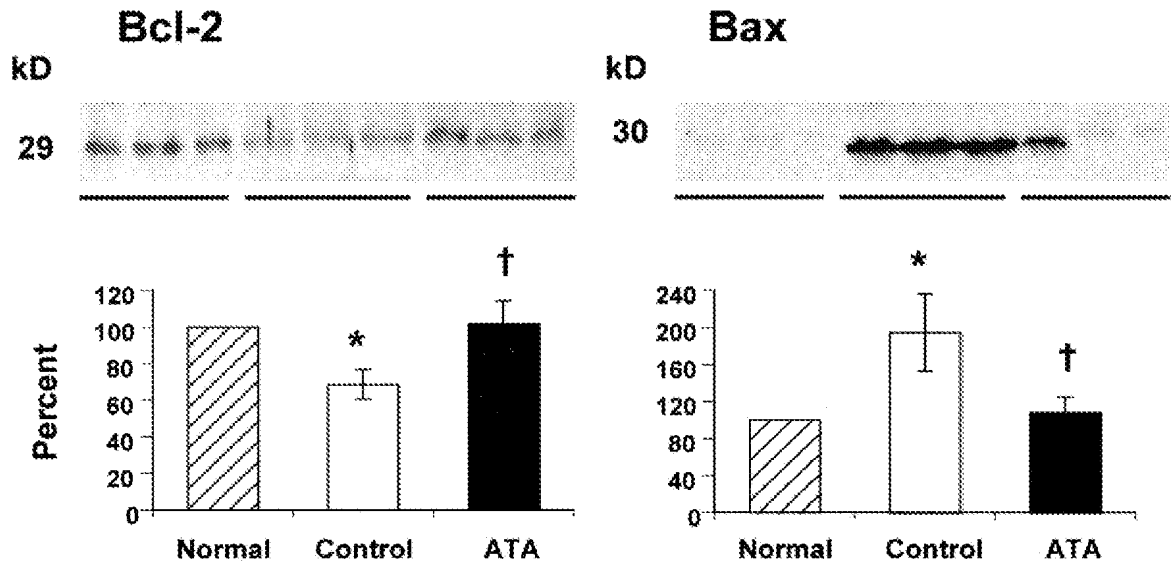

Fig. 5. Expression in Bcl-2 and Bax proteins was visualized by Western blot analysis in non-ischemic and ischemic tissue after 1 h ischemia followed by 24 h of R. Densitometrically, ATA treatment up-regulated expression of Bcl-2 and down-regulated expression of Bax compared with the control group. *$P<0.05$ vs. non-ischemic tissue; †$P<0.01$ vs. control.

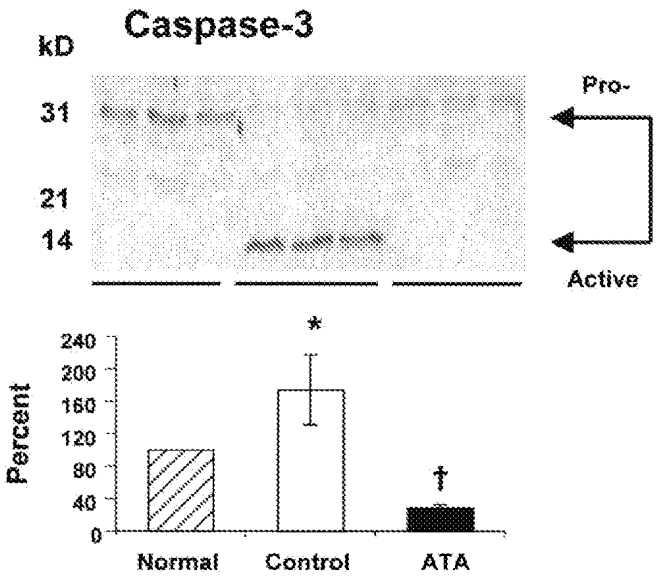

Fig. 6. Caspase-3 activity was visualized by Western blot analysis in non-ischemic and ischemic zones after 1 h ischemia followed by 24 h of R. Procaspase-3 was present in normal tissue in both groups. Cleavage of procaspase-3 level after ischemia and R was significantly inhibited by ATA treatment compared with the control group. *$P<0.05$ vs. non-ischemic tissue; †$P<0.01$ vs. control.

inhibited expression of both pro- and active caspase-3. Opening chest without coronary occlusion did not cause any change in expression of Bcl-2, Bax and caspase-3 activity in the sham group, which were comparable to that in non-ischemic tissue in the control and ATA groups.

3.8. Regional myocardial blood flow at area at risk myocardium

Distribution of myocardial blood flow to the non-ischemic and ischemic epi- and endo-myocardium is shown in Fig. 7. Blood flow in the non-ischemic myocardium remained unchanged during the course of the experiment. Coronary occlusion significantly reduced blood flow in ischemic epi- and endo-myocardium in the two groups from their respective baseline values. Comparable values in regional blood flow in ischemic myocardium in two groups during ischemia suggested that the effect of ATA on infarct size is not related to group difference in collateral blood flow. Release of the coronary snare resulted in a significant increase in blood flow in the ischemic epi- and endo-myocardium in both groups at 15 min of R relative to values at ischemia. ATA administration at R did not change blood flow in ischemic epi- and endo-myocardium at 15 min of R relative to the control group. However, at 24 h of R, blood flow in these zones was significantly greater in ATA-treated hearts. There was no difference in blood flow during the course of the experiment in the sham group, which was comparable to that in non-ischemic tissue in the control and ATA groups (Fig. 7A).

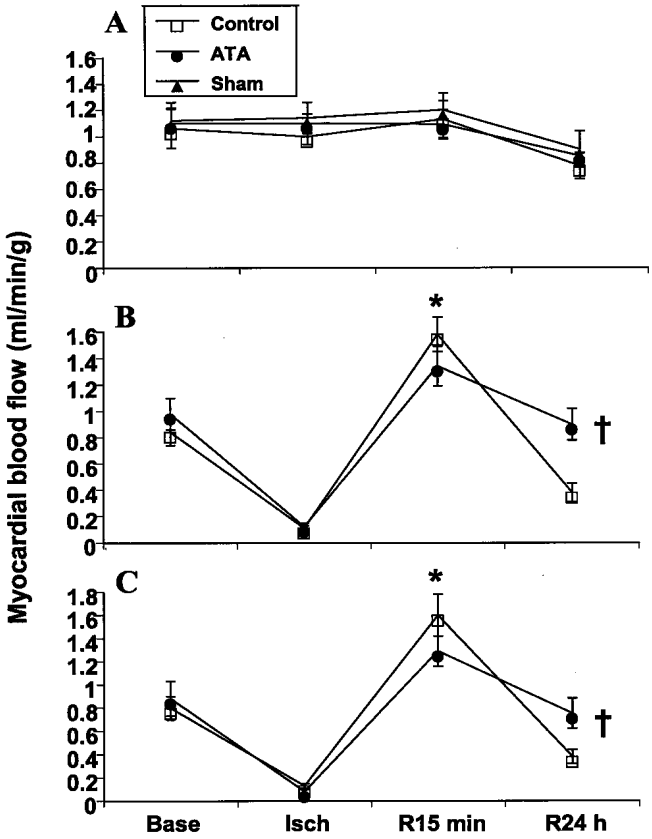

Fig. 7. Change in regional myocardial blood flow in non-ischemic zone (A), ischemic subepicardial- (B) and subendocardial- (C) myocardium during the course of experiment in two groups. Opening chest without coronary occlusion in the sham group did not alter myocardial blood flow (A). *$P<0.001$ vs. baseline. †$P<0.05$ ATA vs. control.

3.9. Coronary artery relaxation after ischemia and R

Ischemia and R significantly blunted the endothelium-dependent and muscarinic receptor-mediated vasorelaxation to acetylcholine (Ach) in the LAD, with a rightward shift of the concentration–response curve and a decrease in maximum relaxation compared with that in the LCX (Fig. 8). ATA treatment at R showed significantly greater in maximum vasodilator response in the LAD than that in the control group. Surgical operation without ischemia did not alter vasodilator response of the LAD to Ach in the sham group (Fig. 8). Ischemia and R did not modify the maximal responses of the LAD or the LCX to the endothelium-independent smooth muscle vasodilator, nitroprusside (data not shown).

3.9.1. MPO activity in ischemic/reperfused myocardium

Very low level of tissue MPO activity, a marker of neutrophil accumulation, was detected in the non-ischemic tissue in both groups. However, ischemia and R significantly increased MPO activity (ΔAbs/min/g) in the area at risk myocardium in the control group (138±28 vs. 3±0.4 in non-ischemic zone, $P<0.01$). This increase in

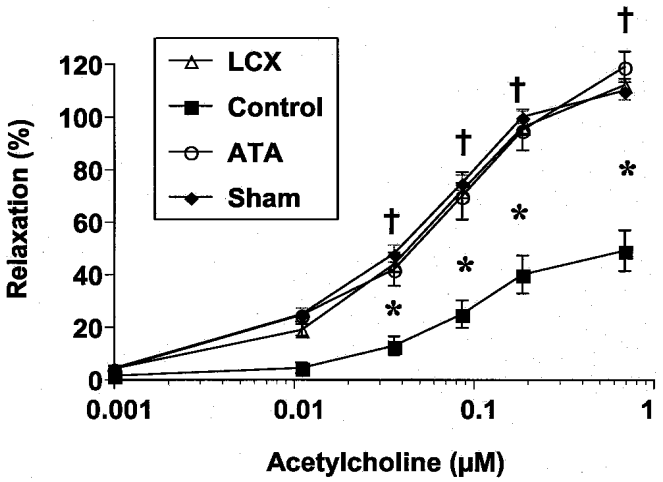

Fig. 8. Dose response of endothelium-dependent vascular relaxation in normal left circumflex (LCX) and ischemic coronary artery rings (LAD) after 1 h coronary occlusion and 24 h of reperfusion. ATA treatment fully recovered vascular relaxation at 24 h of reperfusion compared with the control group. Opening chest without coronary occlusion in the sham group did not change vascular relaxation. Values are mean±S.E.M. of at least 15 rings from five dogs. *$P<0.01$ control vs. LCX; †$P<0.01$ ATA vs. control.

MPO activity was significantly reduced by ATA treatment at R (18±4, $P<0.01$ vs. control group). MPO activity in the myocardium in the sham group (4±0.5) was comparable to that in non-ischemic tissue in the control (3±0.4) and ATA (4±0.1) groups at the end of the experiment.

4. Discussion

Consistent with the changes in anti- and pro-apoptotic proteins, Bcl-2 and Bax, pro-caspase-3 and DNA fragmentation, myocardial apoptosis detected with TUNEL staining in the peri-necrotic myocardium was significantly increased at 24 h of R. ATA given during R reduced the number of TUNEL-positive cells, up- and down-regulated Bcl-2 and Bax expression as well as attenuated caspase-3 activity. With inhibition of TUNEL-positive cells in the ATA group, infarct size was reduced and regional contractile and endothelial function were improved, suggesting a role of apoptosis in contribution of myocardial injury after ischemia and R.

4.1. Inhibition of myocardial apoptosis by ATA administration

ATA, a well-known endonuclease inhibitor with multiple mechanisms of action in different targets, has been now used as a pharmacological tool to detect if cell death, in the organs studied, was due to apoptosis [11,12,14,16]. The application of ATA in various biological studies has shown a profound inhibition in DNA fragmentation, initiation of protein synthesis and transcription, and formation of the ribosomal complex as well as the association of any nucleic acid binding protein with nucleic acid [14,17]. Recent evidence suggests that ATA might be membrane impermeant due to its polyanionic nature, and that the sites of its action are at the cell surface through interacting with receptors rather than on endonuclease in the nucleus [18]. In cultured cell preparations, ATA dose-dependently reduced the number of apoptotic cells initiated by diverse signal pathways including growth factor withdrawal and stimulation with fas ligand, TNFα and caspase [14,18]. Through inhibiting induction of apoptosis, in vivo studies have shown that ATA administered before the ischemic insult reduced infarct size in brain following transient bilateral carotid artery occlusion in gerbils [12], and also protected retinal cells from delayed apoptosis when this compound was given even 6 h after ischemia [16]. ATA could be cardioprotective not only because it inhibits different subtypes of endogenous endonucleases that lead to internucleosomal DNA fragmentation and formation of apoptotic bodies [11], but also because it could regulate protein synthesis involved in regulation of apoptosis as shown previously [13] as well as in the present study. The treatment with ATA at R significantly up-regulated Bcl-2 and down-regulated Bax, and inhibited activation of downstream caspase-3. These alterations corresponded to a reduction in the number of apoptotic cells in the peri-necrotic myocardium, supporting that cardiomyocytes after ischemia and R die, at least in part, by an apoptotic pathway. The selected ATA dose used in the present study was based on a previous in vivo study that showed ATA at 1 mg/kg/h significantly inhibited platelet activation and adhesion to damaged vascular endothelium [19].

4.2. The role of myocardial apoptosis in extension of infarct size during reperfusion

R-induced or accelerated myocardial apoptosis has been well confirmed in different animal species and human [3,4,20,21]. A cross-over of apoptosis to necrosis during reperfusion has been proposed to be in part responsible for extension of infarction over time after the onset of R [10,22,23]. Consistent with studies that showed a delayed apoptosis in late phase of R in liver and brain after transient ischemia [12,24], we have previously reported that after a fixed ischemic event, the number of apoptotic cells in the peri-necrotic myocardium progressively increased during the extended R, suggesting a potential role of apoptosis in development of infarction [10]. Lack in detection of TUNEL-positive cells from TTC-stained myocardium as described previously [7] may help to explain that a cross-over of apoptosis to necrosis during R is partially responsible for extension of infarction over time after the onset of R [10,23]. This dynamic change in apoptosis during R may offer a 'window of opportunity' to

reduce myocardial injury by interrupting the apoptotic process. In this regard, some recent studies have shown a reduction in infarct size by inhibiting development of apoptosis in the peri-necrotic tissue in the heart and brain in an in vivo rat model of acute ischemia and R [22]. Consistent with these previous reports, in the present study, we found that ATA treatment at the onset of R significantly reduced infarct size at 24 h of R by reducing the number of apoptotic cells in the peri-necrotic myocardium, providing direct evidence that limitation in extension of infarct size can be achieved by anti-apoptotic therapy when it is only applied at the beginning of R.

4.3. An improvement in regional contractile dysfunction with ATA during reperfusion

As it has been reported, no recovery of regional contractile dysfunction may be observed after R in a model that results in substantial amounts of necrosis in the area at risk myocardium or modest recovery may require days or months [25]. The degree of dysfunction largely depends upon the transmural extent of infarction within the risk regions [26]. Although a number of potential mechanisms underlying myocardial contractile dysfunction such as neutrophil activation [27], oxygen-derived free radical generation [28] and Ca^{2+} overload [29] have been proposed, evidence has recently shown that cardiomyocyte loss is also a critical factor in induction of left ventricular dysfunction after myocardial ischemia and R [30,31]. Searching for the time course of apoptosis during R, we have previously demonstrated that increasing numbers of apoptotic cardiomyocytes were observed from 6 to 24 h after 60 min of ischemia when regional contractile dysfunction persisted, suggesting that progressively developed apoptosis during R is somehow consistent with the time course of no functional recovery seen at the late stage of R [10,32]. In the present study, an inhibition of apoptosis by ATA significantly improved regional contractile function at 24 h of R, suggesting that apoptotic cell death detected from the peri-necrotic zone may in part participate in subsequent development of intractable left ventricular dysfunction following ischemia and R. These results corresponded to previous animal and clinical studies that showed the correlation between apoptosis and cardiac function as well as the recovery in dysfunction by inhibiting apoptosis [31,32]. Such a phenomenon may be critical for the heart with a larger portion of infarction because additional cardiomyocyte cell death may be decisive for loss of cardiac contractile reserve and induction of heart failure by the dynamic nature of its development [8,26]. However, the partial recovery in regional contractile function after 24 h of R in the ATA group may also suggest that some other factors, in addition to apoptotic cell death, are involved, which are not addressed by anti-apoptotic therapy [25].

4.4. Preservation of vascular endothelial function and regional myocardial blood flow with ATA during reperfusion

The recovery of myocardial function and limitation of infarction during R is dependent, in part, on a patent, functioning vasculature to restore an adequate myocardial blood supply to the ischemic/reperfused region. However, it has been well demonstrated that even after a short period of ischemia, abrupt restoration of blood flow to the ischemic myocardium can cause apparent vascular endothelial cell injury and thereby reduce blood flow supply to the ischemic region, resulting in the 'low-flow' or 'no-reflow' phenomenon. Cell–cell interactions between inflammatory cells (i.e. neutrophils) and vascular endothelium, reduction in local concentration of endogenously released adenosine and nitric oxide, and endothelial cell swelling have been proposed as the potential mechanisms involved [33–36]. Recently, an induction of apoptotic endothelial cells has been reported after a brief period of ischemia followed by R. In the absence of inflammatory–endothelial cell interactions, R-induced vascular endothelial cell apoptosis has been detected in an isolated Langendorff-perfused rat heart after 30 min global ischemia followed by 2 h of R [37]. These authors found that occurrence of vascular endothelial cell apoptosis soon after R precedes cardiomyocyte apoptosis and concluded, therefore, that the release of soluble pro-apoptotic mediators from activated vascular endothelial cells may be the initial cause for the delayed cardiomyocyte apoptosis [37]. A similar phenomenon has been confirmed in in vivo rat liver of normothermic ischemia and R [24].

In the present study, the vascular endothelial cell injury was manifested as an impaired response to endothelium-dependent vascular relaxation and the 'low-flow' or 'no-reflow' phenomenon was confirmed by reduction in myocardial blood flow following the early phase of reactive hyperemia during R. Limited by the methods used in the present study, we did not directly show that vascular endothelial cells die by an apoptotic pathway; however, a significant improvement in vascular endothelial function and regional myocardial blood flow in the ATA group suggested that endothelial injury and perfusion defect during R may be, at least in part, caused by apoptosis. These data were consistent with previous reports showing that in a pig model of LAD stenosis, regional coronary blood flow reduction significantly correlated with the severity of apoptotic cell death [38], and that ATA protected cultured human endothelial cells from oxidized LDLs-elicited apoptosis and occurrence of postapoptotic necrosis [39]. Therefore, preservation of endothelial cell integrity by ATA may partially explain a reduction in platelet adhesion to the injured vascular endothelium [19] and neutrophil infiltration as measured by MPO activity in the present study. Since administration of ATA in the sham group did not alter myocardial blood flow at 24 h of

R, we could exclude a possibility that an improvement in myocardial blood flow at this time point by ATA was induced by a direct vasodilatory effect. However, future studies to elucidate potential mechanisms underlying an inhibition of inflammatory and endothelial cell–cell interactions as well as a correlation of improvement in myocardial blood flow with recovery in regional contractile function and reduction in infarct size at the late stage of R by ATA are needed.

5. Conclusion

In summary, this study demonstrated that treatment with ATA at R significantly reduced infarct size, preserved regional contractile and vascular endothelial cell function, and improved supply of myocardial blood flow to the ischemic myocardium after coronary occlusion, supporting that apoptotic cell death may be involved in exacerbation of myocardial injury after ischemia and R. These results also provide evidence that in clinical practice, the treatment for patients with thrombolytic therapy, percutaneous transluminal coronary angioplasty or coronary artery bypass graft surgery may thus need to consider an application of anti-apoptotic therapy in order to minimize total cell loss as a consequence of R.

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References

[1] Haunstetter A, Izumo S. Toward antiapoptosis as a new treatment modality. Circ Res 2000;86:371–376.
[2] Buja LM, Entman ML. Modes of myocardial cell injury and cell death in ischemic heart disease. Circulation 1998;98:1355–1357.
[3] Zhao Z-Q, Nakamura M, Wang N-P et al. Reperfusion induces myocardial apoptotic cell death. Cardiovasc Res 2000;45:651–660.
[4] Gottlieb RA, Burleson KO, Kloner RA et al. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994;94:1621–1628.
[5] Freude B, Master TN, Robicsek F et al. Apoptosis is initiated by myocardial ischemia and executed during reperfusion. J Am Coll Cardiol 2000;32:197–208.
[6] Veinot JP, Gattinger DA, Fliss H. Early apoptosis in human myocardial infarcts. Hum Pathol 1997;28:485–492.
[7] Yaoita H, Ogawa K, Maehara K et al. Basic science reports. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. Circulation 1998;97:276–281.
[8] Mocanu MM, Baxter GF, Yellon DM. Caspase inhibition and limitation of myocardial infarct size: protection against lethal reperfusion injury. Br J Pharmacol 2000;130:197–200.
[9] Zhao Z-Q, Nakamura M, Wang N-P et al. Dynamic progression of contractile and endothelial dysfunction and infarct extension in the late phase of reperfusion. J Surg Res 2000;94:1–12.
[10] Zhao Z-Q, Velez DA, Wang N-P et al. Progressively developed myocardial apoptotic cell death during late phase of reperfusion. Apoptosis 2001;6:279–290.
[11] Lam TT, Fu J, Hrynewycz M et al. The effect of aurintricarboxylic acid, an endonuclease inhibitor, on ischemia/reperfusion damage in rat retina. J Ocul Pharmacol Ther 1995;11:253–259.
[12] Rosenbaum DM, D'Amore J, Llena J et al. Pretreatment with intraventricular aurintricarboxylic acid decreases infarct size by inhibiting apoptosis following transient global ischemia in gerbils. Ann Neurol 1998;43:654–660.
[13] Mesner Jr. PW, Bible KC, Martins LM et al. Characterization of caspase processing and activation in HL-60 cell cytosol under cell-free conditions. J Biol Chem 1999;274:22635–22645.
[14] Andrew DJ, Hay AWM, Evans SW. Aurintricarboxylic acid inhibits apoptosis and supports proliferation in a haemopoietic growth-factor dependent myeloid cell line. Immunopharmacology 1999;41:1–10.
[15] Laxson DD, Homans DC, Dai X-Z et al. Oxygen consumption and coronary reactivity in postischemic myocardium. Circ Res 1989;64:9–20.
[16] Rosenbaum DM, Rosenbaum PS, Gupta A et al. Retinal ischemia leads to apoptosis which is ameliorated by aurintricarboxylic acid. Vision Res 1997;37:3445–3451.
[17] Heiduschka P, Thanos S. Aurintricarboxylic acid promotes survival and regeneration of axotomised retinal ganglion cells in vivo. Neuropharmacology 2000;39:889–902.
[18] Berry R, Haimsohn M, Wertheim N et al. Activation of the insulin-like growth factor 1 signaling pathway by the antiapoptotic agents aurintricarboxylic acid and Evans blue. Endocrinology 2001;142:3098–3107.
[19] Matsuno H, Kozawa O, Niwa M et al. Multiple inhibition of platelet activation by aurintricarboxylic acid prevents vascular stenosis after endothelial injury in hamster carotid artery. Thromb Haemost 1998;79:865–871.
[20] Umansky SR, Pisarenko OI, Serebryakova LI et al. Dog cardiomyocyte death induced in vivo by ischemia and reperfusion. BAM 1996;6:227–235.
[21] Fliss H, Gattinger D. Apoptosis in ischemic and reperfused rat myocardium. Circ Res 1996;79:949–956.
[22] Olivetti G, Quaini F, Sala R et al. Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. J Mol Cell Cardiol 1994;28:2005–2016.
[23] Umansky SR, Tomei LD. Apoptosis in the heart. In: Kaufmann SH, editor, Apoptosis. Pharmacological implications and therapeutic opportunities, London: Academic Press, 1997, pp. 383–407.
[24] Kohli V, Selzner M, Madden JF et al. Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia–reperfusion injury in the rat liver. Transplantation 1999;67:1099–1105.
[25] Kloner RA, Jennings RB. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 1. Circulation 2001;104:2981–2989.
[26] Inserte J, Garcia-Dorado D, Ruiz-Meana M et al. Effect of inhibition of Na^+/Ca^{2+} exchanger at the time of myocardial reperfusion on hypercontracture and cell death. Cardiovasc Res 2002;55:739–748.
[27] Heusch G. The relationship between regional blood flow and contractile function in normal, ischemic, and reperfused myocardium. Basic Res Cardiol 1991;86:197–218.
[28] Buckberg GD. Protean causes of myocardial stunning in infants and adults. J Cardiol Surg 1993;8(Suppl):214–219, Review.

[29] Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovasc Res 1998;38:291–300.
[30] Laugwitz KL, Moretti A, Weig HJ et al. Blocking caspase-activated apoptosis improves contractility in failing myocardium. Hum Gene Ther 2001;12:2051–2063.
[31] Sabbah HN, Sharov VG, Gupta RC et al. Chronic therapy with metoprolol attenuates cardiomyocyte apoptosis in dogs with heart failure. J Am Coll Cardiol 2000;36:1698–1705.
[32] Schmitt JP, Schröder J, Schunkert H et al. Role of apoptosis in myocardial stunning after open heart surgery. Ann Thorac Surg 2002;73:1229–1235.
[33] Hearse DJ, Bolli R. Reperfusion induced injury: manifestations, mechanisms, and clinical relevance. Cardiovasc Res 1992;26:101–108, Review.
[34] Kukielka GL, Youker KA, Michael LH et al. Role of early reperfusion in the induction of adhesion molecules and cytokines in previously ischemic myocardium. Mol Cell Biochem 1995;147:5–12.
[35] Mazzoni MC, Schmid-Schönbein GW. Mechanisms and consequences of cell activation in the microcirculation. Cardiovasc Res 1996;32:709–719.
[36] Rochitte CE, Lima JAC, Bluemke DA et al. Magnitude and time course of microvascular obstruction and tissue injury after acute myocardial infarction. Circulation 1998;98:1006–1014.
[37] Scarabelli T, Stephanou A, Rayment N et al. Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia/reperfusion injury. Circulation 2001;104:253–256.
[38] Chen C, Ma L, Linfert DR et al. Myocardial cell death and apoptosis in hibernating myocardium. J Am Coll Cardiol 1997;30:1407–1412.
[39] Escargueil-Blanc I, Meilhac O, Pieraggi M-T et al. Oxidized LDLs induce massive apoptosis of cultured human endothelial cells through a calcium-dependent pathway. Prevention by aurintricarboxylic acid. Arterioscler Thromb Biol 1997;17:331–339.